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BIOLOGICALLY ACTIVE EPH FAMILY LIGANDS

INTRODUCTION

The present invention provides for a novel ligand that binds proteins belonging to the Eph subfamily of receptorlike protein tyrosine kinases, such as the Elk receptor and methods for making soluble forms of this ligand that are biologically active.

BACKGROUND OF THE INVENTION

The ability of polypeptide ligands to bind cells and thereby elicit a phenotypic response such as cell growth, survival or differentiation is often mediated through transmembrane tyrosine kinases. The extracellular portion of each receptor tyrosine kinase (RTK) is generally the most distinctive portion of the molecule, as it provides the protein with its ligand-recognizing characteristic. Binding of a ligand to the extracellular domain results in signal transduction via an intracellular tyrosine kinase catalytic domain which transmits a biological signal to intracellular target proteins. The particular array of sequence motifs of this cytoplasmic, catalytic domain determines its access to potential kinase substrates (Mohammadi, et al.,1990, Mol. Cell. Biol., 11: 5068-5078; Fantl, et al., 1992, Cell, 69:413-413).

RTKs appear to undergo dimerization or some related conformational change following ligand binding (Schlessinger, J., 1988, Trend Biochem. Sci. 13:443-447; Ullrich and Schlessinger,

1990, Cell, 61:203-212; Schlessinger and Ullrich, 1992, Neuron 9:383-391); molecular interactions between dimerizing cytoplasmic domains lead to activation of kinase function. In some instances, such as the growth factor platelet derived growth factor (PDGF), the ligand is a dimer that binds two receptor molecules (Hart, et al. , 1988, Science, 240: 1529-1531; Heldin, 1989, J. Biol. Chem. 264:8905-8912) while, for example, in the case of EGF, the ligand is a monomer (Weber, et al., 1984, J. Biol. Chem., 259:14631-14636).

The tissue distribution of a particular tyrosine kinase receptor within higher organisms provides relevant data as to the biological function of the receptor. The tyrosine kinase receptors for some growth and differentiation factors, such as fibroblast growth factor (FGF) are widely expressed and therefore appear to play some general role in tissue growth and maintenance. Members of the Trk RTK family (Glass & Yancopoulos, 1993, Trends in Cell Biol, 3:262-268) of receptors are more generally limited to cells of the nervous system, and the Nerve Growth Factor family consisting of NGF, BDNF, NT-3 and NT-4/5 (known as the neurotrophins) which bind these receptors promote the differentiation of diverse groups of neurons in the brain and periphery (Lindsay, R. M, 1993, in Neurotrophic Factors, S.E. Loughlin & J.H. Fallon, eds., pp. 257-284 (San Diego, CA: Academic Press). The localization of one such Trk family receptor, trkB, in tissue provided some insight into the potential biological role of this receptor, as well as the ligands that bind this receptor (referred to herein as cognates). Thus, for example, in adult mice, trkB was found to be preferentially

expressed in brain tissue, although significant levels of trkB mRNAs were also observed in lung, muscle, and ovaries. Further, trkB transcripts were detected in mid and late gestation embryos. In situ hybridization analysis of 14 and 18 day old mouse embryos indicated that trkB transcripts were localized in the central and peripheral nervous systems, including brain, spinal cord, spinal and cranial ganglia, paravertebral trunk of the sympathetic nervous system and various innervation pathways, suggesting that the trkB gene product may be a receptor involved in neurogenesis and early neural development as well as play a role in the adult nervous system.

The cellular environment in which an RTK is expressed may influence the biological response exhibited upon binding of a ligand to the receptor. Thus, for example, when a neuronal cell expressing a Trk receptor is exposed to a neurotrophin which binds that receptor, neuronal survival and differentiation results. When the same receptor is expressed by a fibroblast, exposure to the neurotrophin results in proliferation of the fibroblast (Glass, et al., 1991, Cell 66:405-413). Thus, it appears that the extracellular domain provides the determining factor as to the ligand specificity, and once signal transduction is initiated the cellular environment will determine the phenotypic outcome of that signal transduction.

A number of RTK families have been identified based on sequence homologies in their intracellular domain. The receptor and signal transduction pathways utilized by NGF involves the product of the trk proto-oncogene (Kaplan et al., 1991, Nature 350:156-160; Klein et al., 1991, Cell 65:189-197). Klein et al. (1989, EMBO J.

8:3701-3709) reported the isolation of trkB, which encodes a second member of the tyrosine protein kinase family of receptors found to be highly related to the human trk protooncogene. TrkB binds and mediates the functional responses to BDNF, NT-4, and, to a lesser extent, NT-3 (Squinto, et al., 1991, Cell 65:885-903; Ip, et al., 1992, Proc. Natl. Acad. Sci. U.S.A. 89:3060-3064; Klein, et al., 1992, Neuron, 8:947-956). At the amino acid level, the products of trk and trkB were found to share 57 percent homology in their extracellular regions, including 9 of the 11 cysteines present in trk. This homology was found to increase to 88 percent within their respective tyrosine kinase catalytic domains. The Trk gene family has now been expanded to include the trkC locus, with NT-3 having been identified as the preferred ligand for trkC (Lamballe, et al., 1991, Cell 66: 967-979; Valenzuela, et al. 1993, Neuron 10:963-974).

The Eph-related transmembrane tyrosine kinases comprise the largest known family of receptor-like tyrosine kinases, with many members displaying specific expression in the developing and adult nervous system. Two novel members of the Eph RTK family, termed Ehk (eph homology kinase) -1 and -2 were identified using a polymerase chain reaction (PCR)-based screen of genes expressed in brain (Maisonpierre, et al. 1993, Oncogene 8:3277-388). These genes appear to be expressed exclusively in the nervous system, with Ehk-1 expression beginning early in neural development. Recently, a new member of this group of related receptors, Ehk-3 has been cloned (Valenzuela, et al. 1995, Oncogene 10:1573-1580).

The elk gene encodes a receptorlike protein-tyrosine kinase that also belongs to the eph subfamily, and which is expressed almost exclusively in the brain (and at lower levels in the testes) (Letwin, et al. 1988; Oncogene 3:621-678; Lhotak, et al., 1991 Mol. Cell. Biol. 11: 2496-2502). Based on its expression profile, the Elk receptor and its cognate ligand are expected to play a role in cell to cell interactions in the nervous system. Other members of the Eph family of receptors that fall within the same subclass as Elk include the Nuk/Cek5, Hek2/Sek4 and Htk receptors (Brambilla and Klein, 1995, Mol. Cell. Neurosci., 6:487-495, Gale, et al., 1996, Neuron 17:9-19).

Unlike the Ehks and Elk receptors, the closely related Eck receptor appears to function in a more pleiotropic manner; it has been identified in neural, epithelial and skeletal tissues and it appears to be involved in the gastrulation, craniofacial, and limb bud sites of pattern formation in the mouse embryo (Ganju, et al. 1994, Oncogene 9:1613-1624).

The identification of a large number of receptor tyrosine kinases has far exceeded the identification of their cognate ligands. At best, determination of the tissues in which such receptors are expressed provides insight into the regulation of the growth, proliferation and regeneration of cells in target tissues. Because RTKs appear to mediate a number of important functions during development, their cognate ligands will inevitably play a crucial role in development.

Although a number of schemes have been devised for the identification of cognate ligands for the many orphan receptors that have been identified, very few such ligands have been identified, and the ligands that have been identified to date appear to have no activity other than the ability to bind their cognate receptor. For example, International Publication Number WO/94/11020 published on May 26, 1994 describes ligands that bind to the Eck receptor. In particular the ligand EBP (also known as B61) is described. However, although binding of B61 to the Eck receptor is disclosed, no biological activity is described. Similarly, despite the description in PCT Publication Number WO94/11384 (published May 26, 1994) of a ligand that binds the Elk receptor, no biological activity was observed, regardless of whether the ligand was presented as membrane bound or in the form of an Fc dimer of the soluble ligand. With respect to the Elk receptor, however, chimeric EGFR-Elk receptors (having the extracellular domain of the EGFR fused to the Elk cytoplasmic domain) have been used to demonstrate the functional integrity (as measured by EGF-stimulated autophosphorylation) of the enzymatic domain of this receptor. (Lhotak and Pawson, 1993, Mol. Cell. Biol. 13:7071-7079).

SUMMARY OF THE INVENTION

The present invention provides for a novel polypeptide ligand, designated as Efl-6, that binds to the Elk, Nuk/Cek5, Hek2/Sek4, Htk, and Sek1 receptors on cells. More importantly, the

invention provides a means of making biologically active, soluble forms of this ligand, which are useful in promoting a differential function and/or influencing the phenotype, such as growth and/or proliferation, of receptor bearing cells. The invention also provides for nucleic acids encoding such polypeptide ligands, and both prokaryotic and eukaryotic expression systems for producing such proteins. The invention also provides for antibodies to these ligands.

According to the invention, soluble forms of the ligands described herein may be used to promote biological responses in Elk, Nuk/Cek5, Hek2/Sek4, Htk, and Sek1 receptor-expressing cells. In particular, a general method is described herein which produces "clustering" of ligands for eph-related receptors, which functions to make otherwise inactive soluble ligands biologically active, or which enhances the biological activity of ligands that, absent such clustering, would have only low levels of biological activity.

The ligands described herein also have diagnostic utilities. In particular embodiments of the invention, methods of detecting aberrancies in their function or expression may be used in the diagnosis of neurological or other disorders.

In other embodiments, manipulation of the interaction between the ligands and their cognate receptor may be used in assay systems designed to identify both agonists and antagonists of Eph receptor ligands. Such agonists and antagonists may be developed for use in the eventual treatment of neurological or other disorders.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Nucleotide and encoded protein sequence of Efl-6. The putative signal sequence is encoded by about nucleotide 202 to about nucleotide 273. The coding region of the mature protein begins at about nucleotide 274 and ends at about nucleotide 1224. The deposited clone has an A at position 698. This change created an amino acid change from Q (Gln) to R (Arg). The coding region for the putative transmembrane domain is shown underlined. The amino acid sequence of the encoded extracellular domain, which is encoded by about nucleotide 274 to about nucleotide 873, is shown in bold letters.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides for a novel polypeptide ligand that binds to the Elk receptor. The novel polypeptide ligand of the present invention is also able to bind other members of the Elk subclass of Eph receptors, including Nuk/Cek5, Hek2/Sek4 and Htk, as well as the only receptor known to "cross subclasses", known as Sek1 (Brambilla and Klein, 1995, Mol. Cell. Neurosci., 6:487-495, Gale, et al., 1996, Neuron 17:9-19). Accordingly, as used herein, the "Elk" receptor refers to Elk, as well as the above receptors known to bind the Elk ligands.

The invention further provides a means of making biologically active, soluble forms of the Efl-6 ligand, which are

useful in promoting a differential function and/or influencing the phenotype, such as growth and/or proliferation, of receptor bearing cells. The invention also provides for nucleic acids encoding such a polypeptide ligand, and both prokaryotic and eukaryotic expression systems for producing this protein. The invention also provides for antibodies to this ligand.

The novel ligand described herein is designated as Efl (Eph transmembrane tyrosine kinase family ligands)-6. A deposit designated as pbluescript SK-encoding Efl-6 was made with the American Type Culture Collection on October 19, 1995 and has received accession number 97319.

According to the invention, soluble forms of the Elk ligand (referred to herein as Efl-6) may be used to promote biological responses in Elk receptor-expressing cells. In particular, a general method is described herein which produces "clustering" of Efl-6 ligand which functions to make otherwise inactive soluble ligand biologically active, or which enhances the biological activity of the ligand which, absent such clustering, would have only low levels of biological activity.

The Efl-6 ligand described herein may also have diagnostic utilities. In particular embodiments of the invention, methods of detecting aberrancies in its function or expression may be used in the diagnosis of neurological or other disorders. In other embodiments, manipulation of the interaction between the ligand and

its cognate receptor may be used in the treatment of neurological or other disorders.

When used herein, Efl-6 includes functionally equivalent molecules in which amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Also included within the scope of the invention are proteins or fragments or derivatives thereof which exhibit the same or similar biological activity and derivatives which are differentially modified during or after translation, e.g., by glycosylation, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc.

Cells that express Efl-6 may do so naturally or may be genetically engineered to produce this ligand, as described supra, by transfection, transduction, electroporation, microinjection, via a transgenic animal, etc. of nucleic acid encoding Efl-6 described

herein in a suitable expression vector. A vector containing the cDNA encoding for EFl-6 deposited with the American Type Culture Collection under the terms of the Budapest Treaty on October 19, 1995 as pBluescriptSK-Efl-6 has been given the ATCC designation 97319.

The present invention encompasses the DNA sequence contained in the above deposited plasmid, as well as DNA and RNA sequences that hybridize to the Efl-6 encoding sequence contained therein, under conditions of moderate stringency, as defined in, for example, Sambrook, et al. Molecular Cloning: A Laboratory Manual, 2 ed. Vol. 1, pp. 101-104, Cold Spring Harbor Laboratory Press (1989). Thus, nucleic acids contemplated by the invention include the sequence as contained in the deposit and as set forth in Figure 1, sequences of nucleic acids that hybridize to such sequence and which bind the Elk receptor, and nucleic acid sequences which are degenerate of the above sequences as a result of the genetic code, but which encode ligand(s) that bind the Elk receptor.

In addition, the present invention contemplates use of the ligands described herein in soluble forms, truncated forms, and tagged forms. This includes monomeric forms of the ligand which may bind to the receptor and function as an antagonist.

Any of the methods known to one skilled in the art for the insertion of DNA fragments into a vector may be used to construct expression vectors encoding Efl-6 using appropriate transcriptional/translational control signals and the protein coding

sequences. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination). Expression of nucleic acid sequence encoding the Efl-6 or peptide fragments thereof may be regulated by a second nucleic acid sequence so that the protein or peptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of the Efl-6 described herein may be controlled by any promoter/enhancer element known in the art. Promoters which may be used to control expression of the ligands include, but are not limited to the long terminal repeat as described in Squinto et al., (1991, Cell 65:1-20); the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the CMV promoter, the M-MuLV 5' terminal repeat the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:144-1445), the regulatory sequences of the metallothioein gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25), see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADH (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal

transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift, et al., 1984, Cell 38:639-646; Ornitz, et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl, et al., 1984, Cell 38:647-658; Adames, et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder, et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert, et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf, et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58); alpha 1-antitrypsin gene control region which is active in the liver (Kelsey, et al, 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram, et al., 1985, Nature 315:338-340; Kollias, et al., 1986, Cell 46:89-94); myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead, et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Shani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

Thus, according to the invention, expression vectors capable of being replicated in a bacterial or eukaryotic host comprising Efl-6 encoding nucleic acid as described herein, are used to transfect the host and thereby direct expression of such nucleic acid to produce the Efl-6 proteins, which may then be recovered in biologically active form. As used herein, a biologically active form includes a form capable of binding to the relevant receptor, such as Elk, and causing a differentiated function and/or influencing the phenotype of the cell expressing the receptor. Such biologically active forms would, for example, induce phosphorylation of the tyrosine kinase domain of the Elk receptor, or stimulation of synthesis of cellular DNA. Alternatively, biologically active Elf-6 ligand includes monomeric forms that bind the receptor and act as antagonists.

Expression vectors containing the gene inserts can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to an inserted *efl* -6 gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign

genes in the vector. For example, if the *efl* -6 gene is inserted within the marker gene sequence of the vector, recombinants containing the insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the *efl -6* gene product, for example, by binding of the ligand to the Elk receptor or portion thereof which may be tagged with, for example, a detectable antibody or portion thereof or binding to antibodies produced against the Efl-6 protein or a portion thereof.

Efl-6 appears to be a conventional transmembrane protein with a cytoplasmic domain. The transmembrane domain is shown underlined in Figure 1. Accordingly, the soluble or extracellular domain of the ligand (sEfl-6) is encoded by the nucleotide sequence from about nucleotide 274 to about nucleotide 873.

The ligands described herein may be produced as membrane bound forms in animal cell expression systems or may be expressed in soluble form. Soluble forms of the ligands may be expressed using methods known to those in the art. A commonly used strategy involves use of oligonucleotide primers, one of which spans the N-terminus of the protein, the other of which spans the region just upstream to a hydrophobic segment of the protein, which represents either the GPI-linkage recognition domain or a transmembrane domain of the protein. The oligonucleotide spanning the C-terminus region is modified so as to contain a stop codon prior to the

hydrophobic domain. The two oligonucleotides are used to amplify a modified version of the gene encoding a protein that is secreted instead of membrane bound. Alternatively, a convenient restriction site in the vector can be used to insert an altered sequence that removes the GPI-linkage recognition domain or transmembrane domain, thus resulting in a vector capable of expressing a secreted form of the protein. The soluble protein so produced would include the region of the protein from the N- terminus to the region preceding the hydrophobic GPI recognition domain or transmembrane domain.

Applicants have discovered that although the soluble ligands produced according to the invention bind to the receptors in the eph subfamily, such soluble ligands often have little or no biological activity. Such soluble ligands are activated, according to the present invention, by ligand "clustering". "Clustering" as used herein refers to any method known to one skilled in the art for creating multimers of the soluble portions of ligands described herein.

In one embodiment, a "clustered" efl-6 is a dimer, made for example, according to the present invention utilizing the Fc domain of IgG (Aruffo, et al., 1991, Cell 67:35-44), which results in the expression of the soluble ligand as a disulfide-linked homodimer. In another embodiment, secreted forms of the ligand are constructed with epitope tags at their C-termini; anti-tag antibodies are then used to aggregate the ligands.

In addition, the invention contemplates other "engineered" ligand molecules that exist as or form multimers. For example, dimers of the extracellular domains may be engineered using leucine zippers. The leucine zipper domains of the human transcription factors c-jun and c-fos have been shown to form stable heterodimers [Busch and Sassone-Corsi, Trends Genetics 6: 36-40 (1990); Gentz, et al., Science 243: 1695-1699 (1989)] with a 1:1 stoichiometry. Although jun-jun homodimers have also been shown to form, they are about 1000-fold less stable than jun-fos heterodimers. Fos-fos homodimers have not been detected. The leucine zipper domain of either c-jun or c-fos are fused in frame at the C-terminus of the soluble or extracellular domains of the above mentioned ligands by genetically engineering chimeric genes. The fusions may be direct or they may employ a flexible linker domain, such as the hinge region of human IgG, or polypeptide linkers consisting of small amino acids such as glycine, serine, threonine or alanine, at various lengths and combinations. Additionally, the chimeric proteins may be tagged by His-His-His-His-His-His (His6), to allow rapid purification by metal-chelate chromatography, and/or by epitopes to which antibodies are available, to allow for detection on western blots, immunoprecipitation, or activity depletion/blocking in bioassays.

Alternatively, multimers may be made by genetically engineering and expressing molecules that consist of the soluble or extracellular portion of the ligand followed by the Fc-domain of hIgG, followed by either the c-jun or the c-fos leucine zippers

described above [Kostelny, et al., J. Immunol. 148: 1547-1553 (1992)]. Since these leucine zippers form predominately heterodimers, they may be used to drive formation of the heterodimers where desired. As for the chimeric proteins described using leucine zippers, these may also be tagged with metal chelates or an epitope. This tagged domain can be used for rapid purification by metal-chelate chromatography, and/or by antibodies, to allow for detection on western blots, immunoprecipitation, or activity depletion/blocking in bioassays.

In another embodiment of the invention, multimeric soluble ligands are prepared by expression as chimeric molecules utilizing flexible linker loops. A DNA construct encoding the chimeric protein is designed such that it expresses two or more soluble or extracellular domains fused together in tandem ("head to head") by a flexible loop. This loop may be entirely artificial (e.g. polyglycine repeats interrupted by serine or threonine at a certain interval) or "borrowed" from naturally occurring proteins (e.g. the hinge region of hIgG). Molecules may be engineered in which the length and composition of the loop is varied, to allow for selection of molecules with desired characteristics. Although not wishing to be bound by theory, applicants believe that membrane attachment of the ligands facilitates ligand clustering, which in turn promotes receptor multimerization and activation. Thus, according to the invention, biological activity of the soluble ligand is achieved by mimicking, in solution, membrane associated ligand clustering. Thus, a biologically active, clustered soluble eph family ligand

comprises (soluble Efl)$_n$, wherein the soluble efl is the extracellular domain of a ligand that binds an eph family receptor and n is 2 or greater. As described herein, Efl-6 is made biologically active according to the process of the invention.

In each case, one skilled in the art will recognize that the success of clustering will require analysis of the biological activity utilizing bioassays such as those described herein. For example, receptor phosphorylation induced by stimulating receptor expressing reporter cells with COS cells overexpressing membrane forms of the ligands, soluble forms of the ligands and clustered ligands may be compared.

Although in some instances dimerization of the ligand is sufficient to induce biological activity, in certain instances, the methods described herein are used to determine the sufficiency of a particular clustering technique. Often dimerization of a soluble ligand utilizing Fc appears to be insufficient for achieving a biological response, yet further clustering of the ligand according to the invention using anti-Fc antibodies may result in a substantial increase in biological activity.

Cells of the present invention may transiently or, preferably, constitutively and permanently express Efl-6 in native form, or in soluble form as tagged Efl-6 or clustered Efl-6 as described herein.

The recombinant factor may be purified by any technique which allows for the subsequent formation of a stable, biologically

active protein. For example, and not by way of limitation, the factor may be recovered from cells either as a soluble protein or as inclusion bodies, from which it may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis. In order to further purify the factor, conventional ion exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used.

In additional embodiments of the invention, recombinant *efl-6* may be used to inactivate or "knock out" the endogenous gene by homologous recombination, and thereby create an Efl-6 protein deficient cell, tissue, or animal. For example, and not by way of limitation, recombinant *efl* may be engineered to contain an insertional mutation, for example the neo gene, which would inactivate the native *efl -6* gene. Such a construct, under the control of a suitable promoter, may be introduced into a cell, such as an embryonic stem cell, by a technique such as transfection, transduction, injection, etc. Cells containing the construct may then be selected by G418 resistance. Cells which lack an intact *efl -6* may then be identified, e.g. by Southern blotting or Northern blotting or assay of expression. Cells lacking an intact *efl -6* may then be fused to early embryo cells to generate transgenic animals deficient in such ligand. A comparison of such an animal with an animal expressing endogenous Efl-6 would aid in the elucidation of the role of the ligands in development and maintenance. Such an animal may be used to define specific neuronal populations, or any other in vivo processes, normally dependent upon the ligand.

The present invention also provides for antibodies to the Efl-6 described herein which are useful for detection of the ligand in, for example, diagnostic applications. Antibodies to the ligand may also be useful for achieving clustering according to the invention. In instances where endogenous ligand exists, the antibody itself may act as the therapeutic by activating existing ligand.

For preparation of monoclonal antibodies directed toward Efl-6, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor, et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, et al., 1985, in "Monoclonal Antibodies and Cancer Therapy," Alan R. Liss, Inc. pp. 77-96) and the like are within the scope of the present invention.

The monoclonal antibodies for diagnostic or therapeutic use may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (e.g., Teng, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:7308-7312; Kozbor, et al., 1983, Immunology Today 4:72-79; Olsson, et al., 1982, Meth. Enzymol. 92:3-16). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human constant regions (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851, Takeda et al., 1985, Nature 314:452).

Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of the Efl-6 described herein. For the production of antibody, various host animals can be immunized by injection with the Efl-6, or a fragment or derivative thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and Corynebacterium parvum.

A molecular clone of an antibody to a selected Efl-6 epitope can be prepared by known techniques. Recombinant DNA methodology (see e.g., Maniatis, et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) may be used to construct nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof.

Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc.

The present invention provides for antibody molecules as well as fragments of such antibody molecules. Antibody fragments which contain the idiotype of the molecule can be generated by

known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

The present invention also provides for methods of treating a patient suffering from a neurological disorder comprising treating the patient with an effective amount of Efl-6, peptide fragments thereof, or derivatives thereof capable of binding to Elk receptor.

The Elk receptor is also expressed primarily in brain. Accordingly, it is believed that the Elk binding ligand described herein will support the induction of a differential function and/or influence the phenotype, such as growth and/or survival of neural cells, expressing this receptor. As described in Gale, et al., 1996, Oncogene 13:1343-1352, Elk-6 (described as Elk ligand 3 in the reference) is notable for its remarkable restricted and prominent expression in the floor plate and roof plate of the developing neural tube and its rhombomere-specific expression in the developing hindbrain. This distribution suggests a role of Efl-6 and its reciprocal receptor, in neuronal guidance and boundary formation, critical features in the organization of the developing vertebrate central nervous system.

The present invention also provides for pharmaceutical compositions comprising the Efl-6 described herein, peptide

fragments thereof, or derivatives in a suitable pharmacologic carrier.

The Efl-6 proteins, peptide fragments, or derivatives may be administered systemically or locally. Any appropriate mode of administration known in the art may be used, including, but not limited to, intravenous, intrathecal, intraarterial, intranasal, oral, subcutaneous, intraperitoneal, or by local injection or surgical implant. Sustained release formulations are also provided for.

As our understanding of neurodegenerative disease/neurotrauma becomes clearer, it may become apparent that it would be beneficial to decrease the effect of endogenous Efl-6. Therefore, in areas of nervous system trauma, it may be desirable to provide Efl-6 antagonists, including, but not limited to, soluble forms of Efl-6 which may compete with cell-bound ligand for interaction with Elk receptor. Alternatively, soluble forms of the Elk receptors (e.g. expressed as "receptorbodies" produced as described herein) may act as antagonists by binding, and thereby inactivating the ligand. It may be desirable to provide such antagonists locally at the injury site rather than systemically. Use of an Efl-6 antagonist providing implant may be desirable.

Alternatively, certain conditions may benefit from an increase in Efl-6 responsiveness. It may therefore be beneficial to increase the number or binding affinity of Efl-6 in patients suffering from such conditions. This could be achieved through gene therapy using either Efl-6, Efl-6 expressing cells, or Elk receptor or receptor chimeras (cells expressing the extracellular domain of the

Elk receptor). Selective expression of such recombinant proteins in appropriate cells could be achieved using their encoding genes controlled by tissue specific or inducible promoters or by producing localized infection with replication defective viruses carrying the recombinant genes.

The Efl-6 encoding DNA as deposited with the ATCC and having accession number 97319 was isolated from a Stratagene (La Jolla, California) human brain (frontal cortex) library (Catalogue No. 936212). The library is in the λZAPII vector. The sequence of the Efl-6 coding region of this vector is set forth in Figure 1.

Assays or purification of the Efl-6 protein may be conducted by use of an Elk receptorbody, which consists of the extracellular domain of Elk fused to the IgG1 constant region. This receptorbody is prepared as follows: The Fc portion of human IgG1, starting from the hinge region and extending to the carboxy terminus of the molecule, was cloned from placental cDNA using PCR with oligonucleotides corresponding to the published sequence of human IgG1. Convenient restriction sites were also incorporated into the oligonucleotides so as to allow cloning of the PCR fragment into an expression vector. Expression vectors containing full length receptors were modified either by restriction enzyme digests or by PCR strategies so as to replace the transmembrane and intracellular domains with restriction sites that allow cloning the human IgG1 fragment into these sites; this was done in such a way as to generate a fusion protein with the receptor ectodomain as its

amino-terminus and the Fc portion of human IgG1 as its carboxy-terminus. An alternative method of preparing receptorbodies is described in Goodwin, et. al. 1993, Cell 73:447-456.

5

DEPOSIT OF MICROORGANISMS

The following vector been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 in accordance with the Budapest Treaty.

10

DEPOSIT	ACCESSION NUMBER
pBluescript SK-Efl-6	97319

15 The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are
20 intended to fall within the scope of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

CLAIMS

1. An isolated and purified nucleic acid molecule encoding Efl-6 protein wherein the sequence of said nucleic acid is selected from the group consisting of:

(a) the sequence of the DNA encoding mature Efl-6 protein contained in the plasmid pBluescriptSK-Efl-6 as deposited with the American Type Culture Collection on October 19, 1995 and designated as 97319;

(b) the sequence of the DNA encoding mature Efl-6 protein as set forth in Figure 1;

(c) DNA sequences that hybridize under moderately stringent conditions to the DNA of (a) or (b) and which encode a protein that binds a receptor belonging to the Elk subfamily of Eph receptors; and

(d) DNA sequences that are degenerate as a result of the genetic code to a DNA sequence of (a), (b), or (c) and which encode an Efl-6 protein that binds a member of the Elk subclass of Eph receptors.

2. Isolated and purified mature Efl-6 protein having an amino acid sequence as set forth in Figure 1.

3. An isolated nucleic acid encoding the extracellular domain of Efl-6 (sEfl-6) having a sequence selected from the following:

(a) the sequence set forth from about nucleotide 274 to about nucleotide 873 of Figure 1; and

(b) a sequence which encodes the extracellular domain of Efl-6 as set forth in Figure 1.

4. Purified sEfl-6 encoded by the nucleotide sequence of claim 3.

5. (sEfl-6)n comprising the sEfl-6 protein according to claim 4, wherein n is 2 or greater.

6. Efl-6 ligandbody comprising soluble Efl-6 protein according to claim 4 and the Fc portion of IgG.

7. A vector which comprises a nucleic acid molecule of claim 1.

8. A vector according to claim 7 wherein the nucleic acid molecule is operatively linked to an expression control sequence capable of directing its expression in a host cell.

9. A host cell containing a vector according to claim 8.

10. A vector which comprises a nucleic acid molecule of claim 3.

11. A vector according to claim 10 wherein the nucleic acid molecule is operatively linked to an expression control sequence capable of directing its expression in a host cell.

12. A host cell containing a vector according to claim 11.

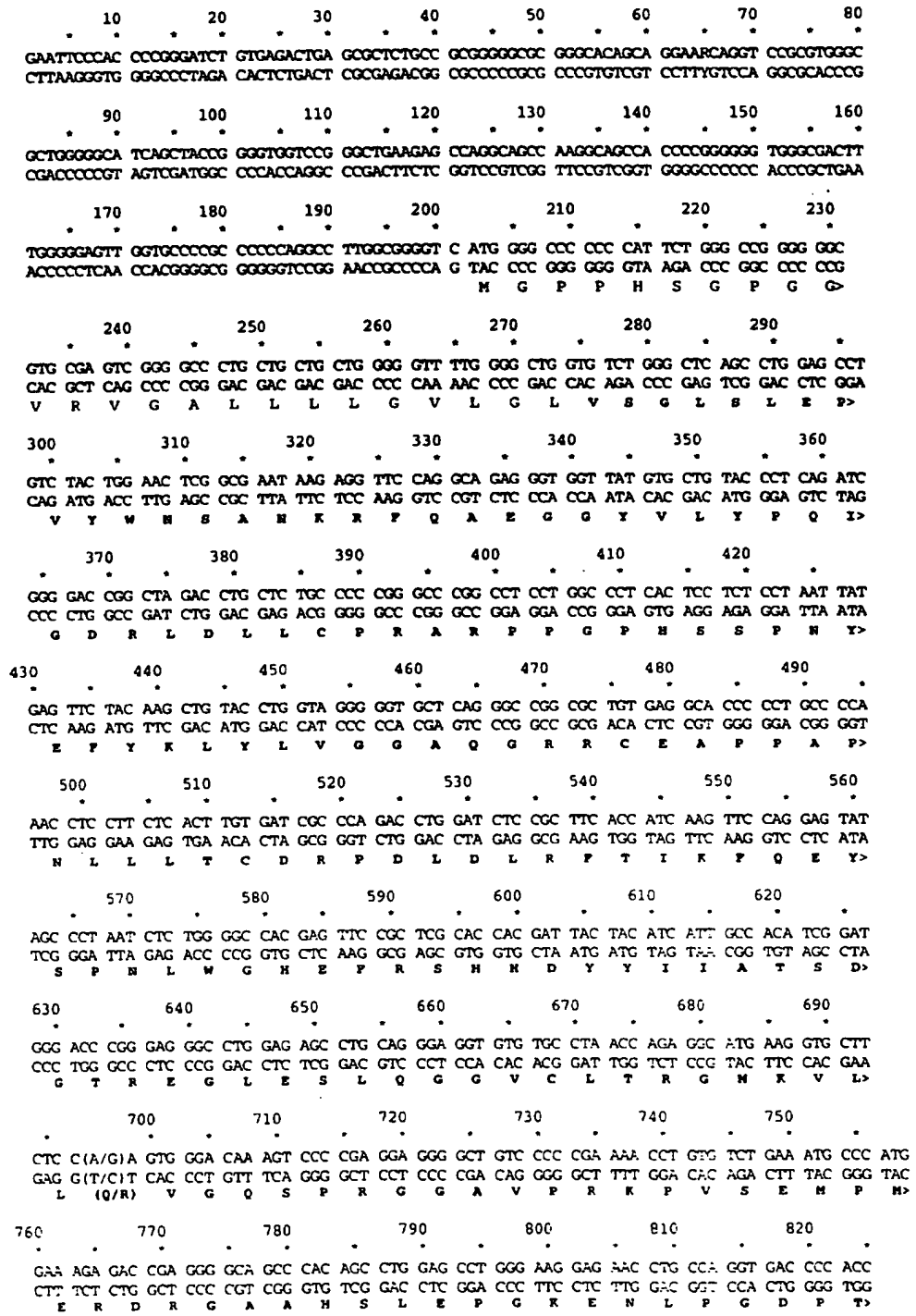
13. A method of producing Efl-6 ligand which comprises growing cells of a host according to claim 8 under conditions permitting production of the ligand, and recovering the ligand so produced.

14. A method of producing Efl-6 soluble ligand which comprises growing cells of a host according to claim 11 under conditions permitting production of the ligand, and recovering the ligand so produced.

15. An antibody which specifically binds the ligand of claim 2 or 4.

16. An antibody according to claim 15 which is a monoclonal antibody.

FIGURE 1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M G P P H S G P G G>
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L (Q/R) V G Q S P R G G A V P R K P V S E M P M>
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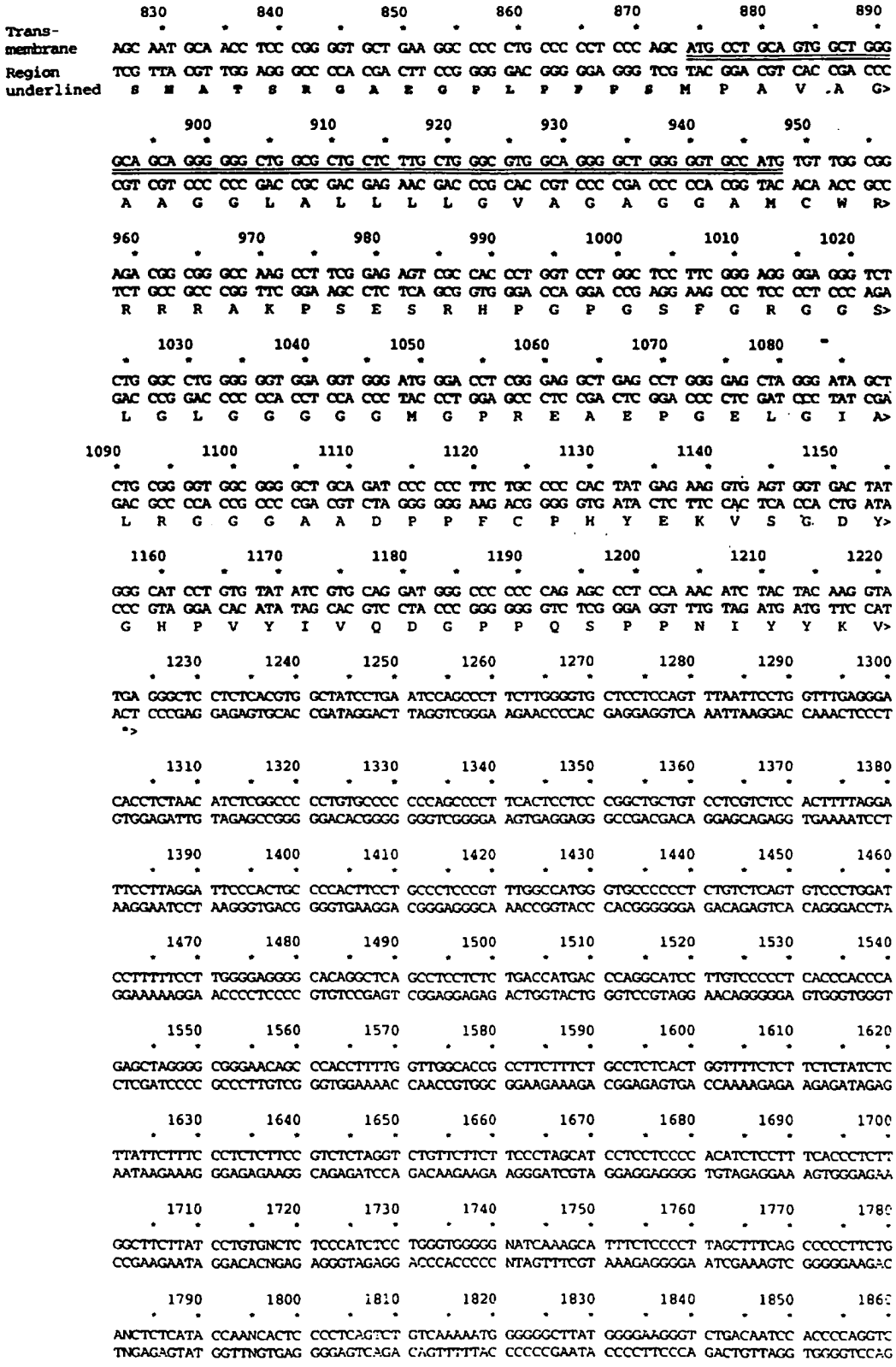
SUBSTITUTE SHEET (RULE 26)

FIGURE 1 (contd)



INTERNATIONAL SEARCH REPORT

Inte nal Application No
PCT/US 96/17201

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/47 C12N5/10 C07K16/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 11384 A (IMMUNEX CORP) 26 May 1994 cited in the application see claims 1-12	1-16

P,X	ONCOGENE, 13 (6). 1996. 1343-1352., XP000644665 GALE N W ET AL: "Elk-L3, a novel transmembrane ligand for the Eph family of receptor tyrosine kinases, expressed in embryonic floor plate, roof plate and hindbrain segments" see the whole document	1-14

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 February 1997	07.03.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Gurdjian, D

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Accession number HSU57001 EMBL/Genbank/DDBJ databases 31-07-96 Cerreti D.P. XP002025757 see the whole document -----	1-5

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int[illegible]onal Application No
PCT/US 96/17201

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9411384	26-05-94	AU-B-	669960	27-06-96
		AU-A-	5667594	08-06-94
		CA-A-	2148484	26-05-94
		EP-A-	0669929	06-09-95
		JP-T-	8503368	16-04-96
		NZ-A-	258697	26-03-96
		US-A-	5512457	30-04-96